Hemin prevents in-stent stenosis in rat and rabbit models by inducing heme-oxygenase-1

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Objective: **The introduction of drug-eluting stents (DES) has largely added benefit to the percutaneous coronary intervention. Questions about the long-term safety of DES have been raised, however, particularly with respect to late stent thrombosis. Research efforts are now being directed toward therapeutics that can impede smooth muscle proliferation and promote vascular healing. Emerging data suggest that heme oxygenase-1 (HO-1), an inducible oxidoreductase enzyme system, can exert cytoprotective effects on endothelial cells and limit smooth muscle cell proliferation. We assessed the ability of hemin, a potent HO-1 inducer, to reduce in-stent stenosis without compromising re-endothelialization.**

Methods: **Rat aorta and rabbit iliac arteries were stented. Animals received ongoing treated with intraperitoneal hemin (50 mg/kg) or vehicle. At 7 to 28 days after surgery, stented arterial segments were collected and processed for histologic, electron microscopy, or protein analysis.**

Results: **In both models, treatment with hemin reduced neointima growth without compromising re-endothelialization of the stented arteries. In the rat aorta, analysis of protein expression at 7 and 28 days after stenting revealed that hemin increased HO-1 expression and limited the early inflammatory, apoptotic, and proliferative cellular events that are common to in-stent stenosis. Hemin treatment decreased the expression of the Ki-67 protein and the activity of key regulators of smooth muscle cell proliferation, including p42/44, RhoA, and up-regulated the expression of cyclin-dependent kinase inhibitors. The beneficial effects of hemin were abolished in the presence of tin-protoporphyrin IX, an HO inhibitor. Finally, treatment with tricarbonylchloro(glycinato)ruthenium(II), a carbon monoxide donor, reduced in-stent stenosis in the rat aorta, suggesting that carbon monoxide, a by-product of heme degradation, might contribute to the protective effect of hemin.**

Conclusion: **These results suggest that HO-1 is important in limiting in-stent stenosis and can be regarded as a new therapeutic target. (J Vasc Surg 2010;51:417-28.)**

Clinical Relevance: **Long-term outcomes of endovascular treatments have not been impressive due to vascular stenosis caused mainly by intimal hyperplasia. Questions have been raised about the long-term safety of drug-eluting stents, particularly with respect to late stent thrombosis. Accumulating data indicate a link between inflammation and in-stent stenosis as well as between delayed endothelialization and thrombosis. We demonstrated in an animal model that hemin, a compound used in the management of porphyria, induced heme oxygenase-1 and limited inflammation and in-stent restenosis without compromising endothelialization. Thus, heme oxygenase-1 may be considered as a novel target in the next generation of drug-eluting stents for preventing in-stent restenosis after endovascular therapies.**

Recurrent stenosis remains the major drawback of percutaneous transluminal angioplasty. In percutaneous coronary intervention (PCI), stenting provides an added benefit by reducing the risk of reocclusion.[1] However, the major adverse event occurring with the use of stent devices is in-stent stenosis (ISS),[2] which is considered a local vascular response to injury. Endothelial injury produces elements of thrombus formation, and fibrin and platelet deposition stimulate inflammatory aggregates. The subsequent production of adhesion molecules, cytokines, and growth factor by the platelets, inflammatory cells, and smooth muscle cells leads to an enhanced inflammatory reaction, apoptosis, and smooth muscle proliferation.

Anticoagulant and antiplatelet therapies have to an extent succeeded in reducing the complications of thrombosis. Furthermore, with the advent of drug-eluting stents (DESs) that can deliver antiproliferative drugs, it has been anticipated that by reducing stenosis, DESs may improve the long-term prognosis of patients treated with these

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devices,[3,4] The demand for these devices in coronary angioplasty has increased progressively, and by 2004, DESs were used in 80% of all PCIs.

Although the short-term to midterm beneficial effect of DESs has been recognized in coronary vessels, recent long-term meta-analyses of DESs have raised questions about their long-term safety, particularly with respect to late stent thrombosis. Poor long-term effects of DESs may arise from the nonselective inhibition of endothelial cell proliferation by the antiproliferative drugs, which could result in delayed and incomplete re-endothelialization as well as incomplete healing of the stented vessels.[5,6] Finally, the use of DESs for noncoronary applications is still investigational, and long-term follow-up results are needed to determine the superiority of DESs.[7]

Research efforts are now being directed toward therapeutics that can impede smooth muscle proliferation and promote vascular healing. Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in heme degradation that leads to the generation of free iron, biliverdin, and carbon monoxide (CO). Accumulated evidence suggests that HO-1 offers protection against several cardiovascular disorders.[8] We recently observed that hemin, a potent HO-1 inducer, prevented arterial thrombosis.[9] The induction of HO-1 has been shown to prevent the proliferation of smooth muscle cells and to promote the growth of endothelial cells in vitro.[10,11] Increased HO-1 expression is associated with decreased intimal thickening after balloon injury.[12-14] However, percutaneous coronary angioplasty is widely associated with stent insertion, thus stenosis therapies must include stenting of vessels.

Taken together, these reports suggest that induction of HO-1 might limit ISS without compromising vessel healing. Among in vivo models of ISS, stenting of the rat aorta has been shown to be a reliable and reproducible model of ISS that, in contrast with other species, enables the investigation of cellular mechanisms.[15]

This study assessed the potential protective effects of hemin against the development of ISS in rat and in hypercholesterolemic rabbit models. In particular, we wanted to test the hypothesis that hemin treatment and the delivery of CO, a by-product of heme degradation by HO-1, would limit excessive neointima formation and allow the endothelialization of the stent struts after stent implantation.

MATERIALS AND METHODS

All animal experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (US National Institutes of Health publication 85-23, revised 1996), in accordance with the regulations of the official edict of the French Ministry of Agriculture, and were approved by local Institutional Animal Care and Use Committee (CL2008-015 and CL2008-028). All surgical procedures were performed by a single operator.

Rat model of in-stent stenosis. Male Wistar rats (Janvier, Le Gesnest-Saint-Isles, France), weighing 350 to 400 g, were randomly separated into control or drug-treated groups. On day 0, the rats were anesthetized and the abdomen was opened by a sagittal incision. Under microscopic view, the abdominal aorta was separated from the vena cava, and small side branches from the aorta were ligated. An arteriotomy was made in the proximal part of the 2.5 cm-long isolated infrarenal aortic segment. Then, a premounted stent bare-metal stent (BMS), 2.5 × 14 to 18 mm, or a sirolimus DES, 2.5 × 14 mm (Cordis, Johnson & Johnson, Miami, Fla) was deployed at its nominal pressure (8 atm).

At 7 and 28 days after stenting, the animals were anesthetized, heparinized, and blood was collected. Stented arteries were perfused with fixative for histologic and electron microscopy analysis (on day 28) or frozen for protein expression analysis (on days 7 and 28).

Rats implanted with BMSs received vehicle, intraperitoneal (IP) hemin (50 mg/kg), tin-protoporphyrin IX (SnPPIX; 15 mg/kg), a potent inhibitor of HO[16]; SnPPIX plus hemin (50 mg/kg each), or CORM-3 (tricarbonylchloro(glycinato)ruthenium[II]; 30 mg/kg), a water soluble CO-releasing molecule.[17] On the basis of preliminary experiments, drugs were administered every 48 hours starting 2 days before surgery. Rats implanted with sirolimus-DESs received saline.

For each series of experiments, different control and drug-treated rats were used and distributed as indicated in the flow chart (Fig 1, *A*). Stent length was homogenous between the different groups to avoid bias.

Hypercholesterolemic rabbit model of ISS. Male New Zealand White rabbits (3.5 to 4.0 kg; INRA, Nouzilly, France) received a hypercholesterolemic diet (standard diet supplemented with 1% cholesterol) starting 4 weeks before surgery and maintained throughout the duration of the experimental protocol. On day 0, the animals were anesthetized and the right common carotid artery was catheterized with a 5F introducer sheath. A 0.035-inch guidewire was used to position a 4F diagnostic catheter in the abdominal aorta under fluoroscopic observation. The iliac arteries were catheterized using a 0.014-inch guidewire, and premounted stents (3.0 × 10 to 24-mm balloon-expandable BMS or sirolimus-DES; Cordis) were deployed at their nominal pressure (8 atm) in each iliac artery under fluoroscopic guidance. The stent-to-artery ratio was oversized (ratio 1.2:1.0).

After stent implantation, vessel patency was documented with angiography. At 28 days after stent implantation, a second angiography was performed under general anesthesia, followed by euthanasia and perfusion-fixation. The stented arteries were collected and processed for histologic or electron microscopic analysis. Control rabbits received vehicle (saline, IP), and the hemin-treated rabbits received hemin (50 mg/kg, IP) every 48 hours, starting 2 days before surgery. In the control group, three rabbits were implanted with a sirolimus-DES in one iliac artery (Fig 1, *B*). Stent length was homogenous between the different groups to avoid bias.

Serum cholesterol levels in the control and hemin-treated rabbits were, respectively, 391 ± 20 and 381 ± 36

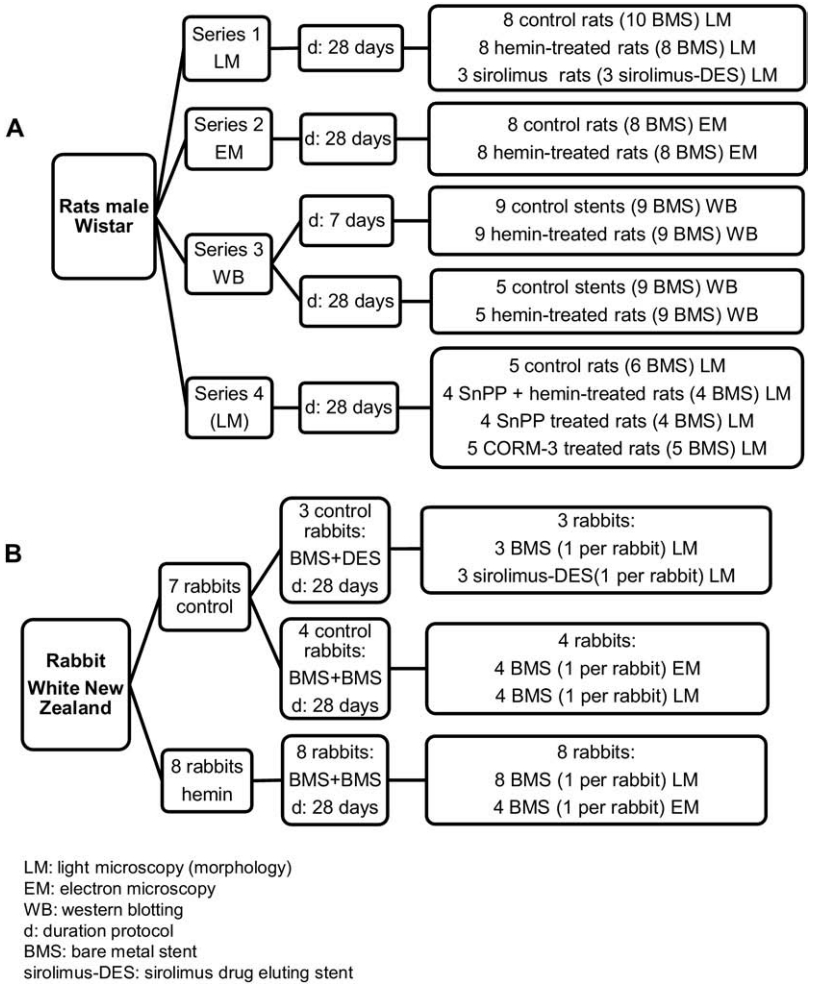

Fig 1. Study flow chart for **(A)** rat model and **(B)** hypercholesteremic rabbit model. *BMS,* Bare-metal stent; *CORM-3,* (tricarbonylchloro(glycinato)ruthenium[II]; *DES,* sirolimus drug-eluting stent; *EM,* electron microscopy; *LM,* light microscopy; *SnPP,* tin-protoporphyrin; *WB,* Western blotting.

mg/dL on day 0 (before surgery, $P < .05$) and 722 ± 40 and 640 ± 38 mg/dL on day 28 ($P < .05$).

Histologic analysis. Stented arteries were fixed in 4% paraformaldehyde phosphate-buffered solution, dehydrated, and embedded in methylmethacrylate polymer (Technovit 9100 new, Heraeus, Germany). Embedded vessels were cut along the entire stented length. Sections (60-μm thick) were stained with Mayer hematoxylin and eosin and viewed under light microscopy. From each stented vessel, 15 to 25 sections randomly chosen along the entire stented length were analyzed by computer-based image analysis using Optimas 6.5 software (Media Cybernetics, Silver Spring, Md). The degree of in-stent stenosis was derived from the ratio of the area of neointima to that of the media (n/m ratio), and the statistical analysis was conducted on the average of all sections per stent. Because measurements were made by a single operator who was blinded to the treatments, we used the standard deviation derived from the average of all sections per stent as an index of the variability of stenosis within each stent.

Electron microscopy. Stented arteries were fixed in 4% paraformaldehyde and 1% glutaraldehyde phosphate-buffered solution (pH 7.2). Then they were cut longitudinally, and one-half was used for transmission electron microscopy (TEM) and the other half for scanning electron microscopy (SEM).

For TEM, samples were embedded in Epon. Ultrathin sections (75 nm) were processed and contrasted with uranyl acetate and lead citrate and examined through a Jeol 1011 electron microscope (Tokyo, Japan) equipped with a Gatan digital camera driven by Digital Micrograph software (Gatan, Pleasanton, Calif).

For immunogold labeling, ultrathin sections were incubated with antiplatelet endothelial cell adhesion molecule-1 goat polyclonal antibody (SantaCruz Biotechnology Inc, Santa Cruz, Calif) and then incubated with a secondary

gold-labeled (15-nm gold particles) goat antirabbit immunoglobulin (Ig) G (Electron Microscopy Science, Euromedex, France). For each stented segment, three stent struts with surrounding neointima were randomly chosen.

For SEM, the samples were processed and coated by platinum sputtering (5 nm) and observed using a LEO DSM 982 SEM (Zeiss, Germany). The coverage of stented vessel was analyzed over the entire surface of the specimen.

Hemin plasmatic assay and bilirubin assay. The plasma concentrations of heme were determined using the pyridine-hemochromogen method, as previously described.[9] Quantitative determination of bilirubin production was determined from the plasma of rats using the Quantichrom Bilirubin Assay kit (Bioassay Systems, Gentaur, France). Briefly, total bilirubin was assayed from 200 μL of plasma using the Jendrassik-Grof method according to the manufacturer's instructions.

Protein expression. Protein expression was determined in the stented rat aorta at 7 and 28 days after stent deployment. Stented aortic segments were placed in lysis buffer, and the stent was carefully removed under optical control. The primary antibodies used were anti-HO-1, anti-p42/44 mitogen-activated protein kinase (MAPK), antiphosphorylated p42/44 MAPK, anti-$p27^{kip1}$ (Ozyme, France), anti-Ki67, anti-p21 (SantaCruz Biotechnology Inc, France), anti-RhoA (Upstate, France) and antiactin (Sigma-Aldrich, France). The secondary antibodies were antirabbit IgG, antimouse IgG, or antigoat horseradish peroxidase-conjugated (Ozyme). Bound antibodies were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) and quantified by densitometric analysis (NIH Image analysis software).

Cytokines array was performed according to the manufacturer's instruction. Briefly, equal amount of proteins (300 μg) were incubated with a detection antibody cocktail for 1 hour at room temperature. Then each sample/antibodies mixture was incubated with a nitrocellulose membrane with spotted antibodies and incubated 2 hours at +6°C (Proteome Profiler Rat Cytokine Array, R&D Systems, France). Bound antibodies were visualized by ECL and quantified by densitometric analysis.

Statistical analysis. Data were reported as mean ± standard error of the mean, and *n* refers to the number of stented arteries from which the analysis was performed. For histologic data, we used an unpaired *t* test to compare means for control and hemin groups. For electron microscopy and Western blot analysis, comparisons of means between the control and hemin groups were performed using an unpaired *t* test. Values of $P < .05$ were considered statistically significant.

RESULTS

Effect of hemin treatment on in-stent stenosis in rat and rabbit models. In control rats, a concentric neointima was observed 28 days after stenting. The ratio of the area of neointima to the area of media (n/m ratio) was 0.83 ± 0.06 (n = 8; Fig 2, *A*). In rats that were implanted with a sirolimus-DES, neointima was almost absent (n/m = 0.45 ± 0.03, n = 3), and the stent struts remained in contact with the lumen. In hemin-treated rats, the n/m ratio was decreased by 30% compared with the control group (0.57 ± 0.03, n = 8, $P < .01$), and the stent struts remained covered by a thin layer of cells (Fig 2, *A*). Furthermore, the variability of the stenosis within each arterial segment was lowered in hemin-treated rats (Fig 2, *A*). In this group, the plasma hemin concentration was 32 ± 3 μmol/L.

At 28 days after stent deployment, angiography of the aortoiliac bifurcation revealed stenosis in four to seven hypercholesterolemic control rabbits (Fig 2, *B*); this was not observed in the hemin-treated rabbits. Histologic analysis of the stented arteries (Fig 2, *B*) revealed that n/m ratio was 0.90 ± 0.05, which was decreased in hemin-treated rabbits (48%, $P < .001$, n = 8) compared with 1.72 ± 0.10 in the controls (n = 7). Similarly, the variability of the stenosis along the stented vessels was reduced in the hemin group (Fig 2, *B*).

Effect of hemin treatment on endothelialization of stented vessels in rat and rabbit models. In the stented rat aorta at 28 days after stenting, TEM analysis revealed a thinner neointima covering the stent struts in the hemin-treated group compared with the control animals (n = 8 each; Fig 3, *A*), which is in agreement with the histologic analysis. The ultrastructure of the cells facing the arterial lumen was similar to that of endothelial cells (Fig 3, *A*). Immunogold labeling of CD31, an endothelial marker, was positive on these cells (Fig 3, *B*). SEM analysis showed that the endothelial coverage area was similar in both control and hemin-treated rats (Fig 3, *C*).

In the hypercholesterolemic rabbits, TEM revealed similar results to those observed in the rats (Fig 3, *D*). In addition, SEM analysis showed similar endothelialization 28 days after surgery in controls and hemin-treated rabbits (n = 4 each, Fig 3, *E*).

Effects of hemin treatment on protein expression in rat. Because the inflammatory response is an early event after stent deployment, we assessed the levels of various cytokines and inflammatory adhesion molecules in stented aortic segments at 7 days after stent deployment. A protein array revealed a significant decrease of several immunogenic and chemoattractant cytokines such as interleukin 6 and tumor necrosis factor-α as well as adhesive molecules such as intercellular adhesion molecule 1 in the hemin-treated rats (n = 4) compared with the control group (n = 4 each, $P < .05$; Fig 4, *A*).

At 7 days after stent deployment, HO-1 expression was significantly increased in hemin-treated rat aortas compared with controls (n = 5 each, $P < .01$), indicating that the hemin treatment was sufficient to induce HO-1 protein (Fig 4, *B* and *C*). Western blotting showed that the level of cleaved caspase 3 was markedly attenuated in the hemin-treated rats ($P < .01$), indicating reduced apoptotic response. In hemin-treated aortas, Ki-67 expression was significantly decreased, indicating reduced proliferation of the vascular cells (Fig 4, *C*).

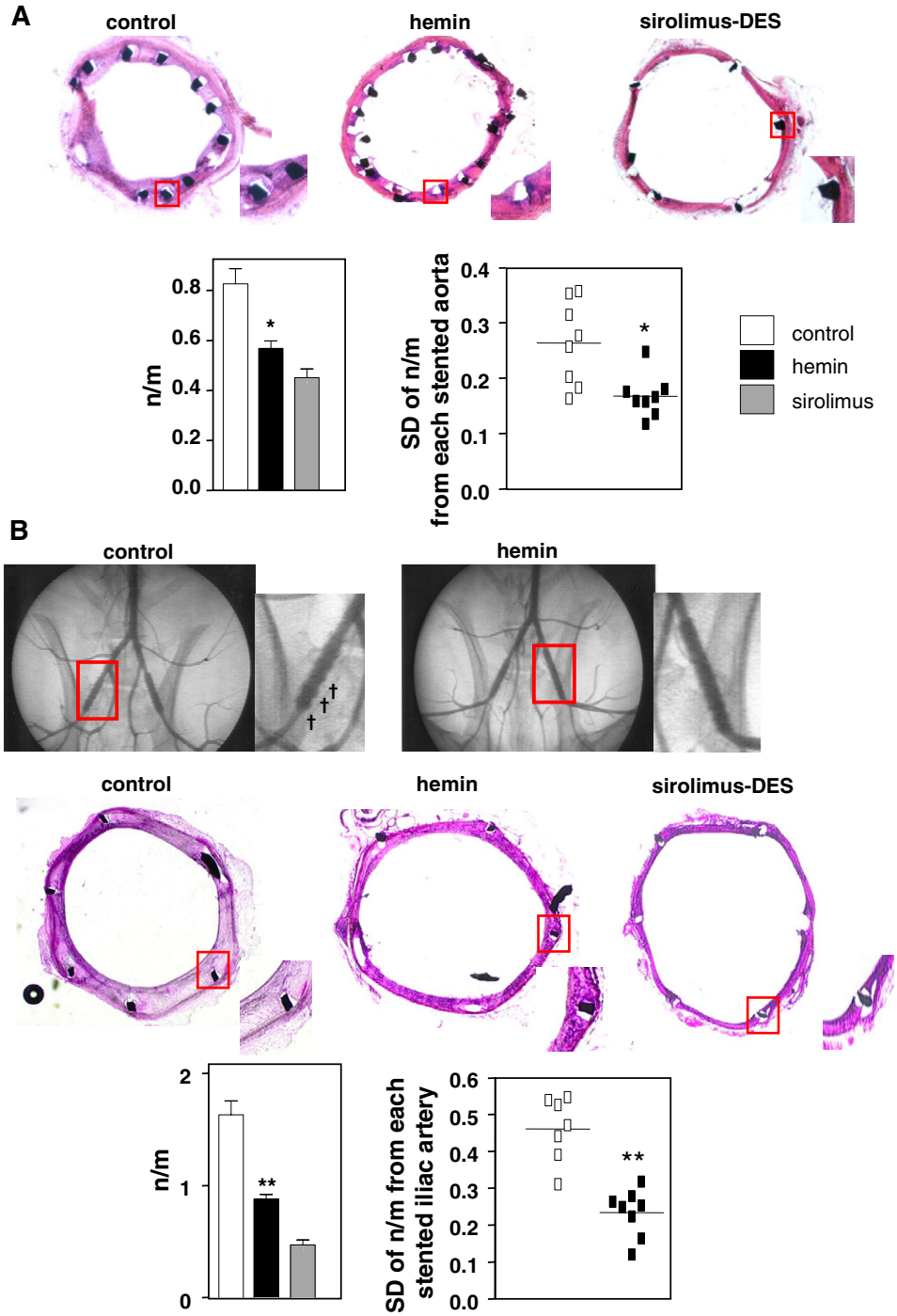

Fig 2. Effect of hemin treatment on in-stent stenosis is shown in rats and hypercholesterolemic rabbits. **A,** Rat aorta morphology 28 days after stenting. The bar graph shows the n/m ratio in control (n = 8), hemin-treated (n = 8), and aortas stented with sirolimus drug-eluting stents (*DES*, n = 3). The scatter graph indicates the standard deviation *(SD)* of the n/m ratio of each stented aortic segment from control and hemin-treated rats. SD was derived from the average of the 15 to 25 analyzed sections per stent and used as an index of the variability of stenosis within each stent. **B,** Angiography of the rabbit aortoiliac bifurcation 28 days after stent deployment is shown in the **top panel**. The insert shows higher magnification of the *red box*. The *daggers* indicate stenosis. The morphology of the iliac stented arteries *(red box)* is presented in the **bottom panel**. The bar graph shows the n/m ratio in control (n = 7), hemin-treated (n = 8), and sirolimus-DES stented iliac arteries (n = 3). The results are the mean ± standard error of the mean. The scatter graph indicates the SD of the n/m ratio of each stented iliac arteries. Significant difference from control: $*P < .05$, $**P < .005$.

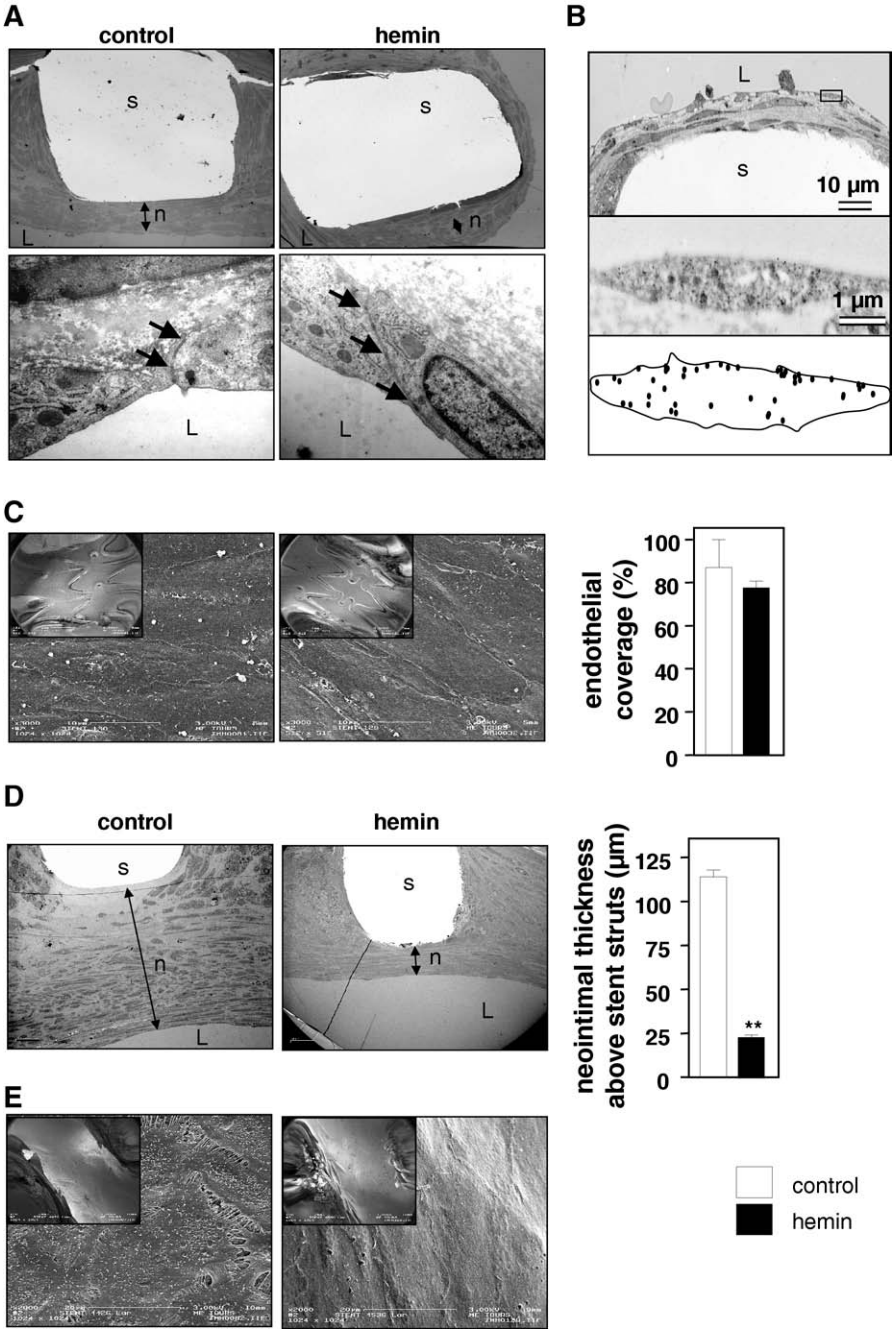

Fig 3. A, Transmission electron microscopic (TEM) analysis of the endothelialization of stented arteries is shows neointima *(n)* around the stent strut *(s)* in the control and hemin-treated rats (original magnification ×875). Higher magnification (×5000) of the cells at the interface of the lumen (*L*) shows ultrastructure similar to that of endothelial cells. The *arrows* indicate tight junctions. **B,** Immunogold labeling of CD31 on cells facing the arterial lumen in hemin-treated rats. The top panel shows neointima above stent strut. Higher magnification of the *black box* is presented below and shows positive labeling of CD31 on the selected cell. The schematic diagram shows a two-dimensional reconstruction of the selected cell from five consecutive ultrathin sections. **C,** Scanning electron microscopy (SEM) shows endothelial coverage of the stented aorta. The inserts show SEM at a lower magnification of the stented arteries. The bar graph shows the mean endothelial coverage in the control and hemin-treated groups (n = 8 per group). The *error bars* show the standard error of the mean. **D,** TEM shows the neointima *(n)* around the stent strut *(s)* in rabbit iliac arteries. The inserts show SEM at a lower magnification of the stented arteries. The bar graph indicates neointima thickness above the stent struts (n = 4 in each groups). The *error bars* show the standard error of the mean. **Significantly different from control ($P < .005$). **E,** SEM shows endothelial coverage of the stented iliac artery.

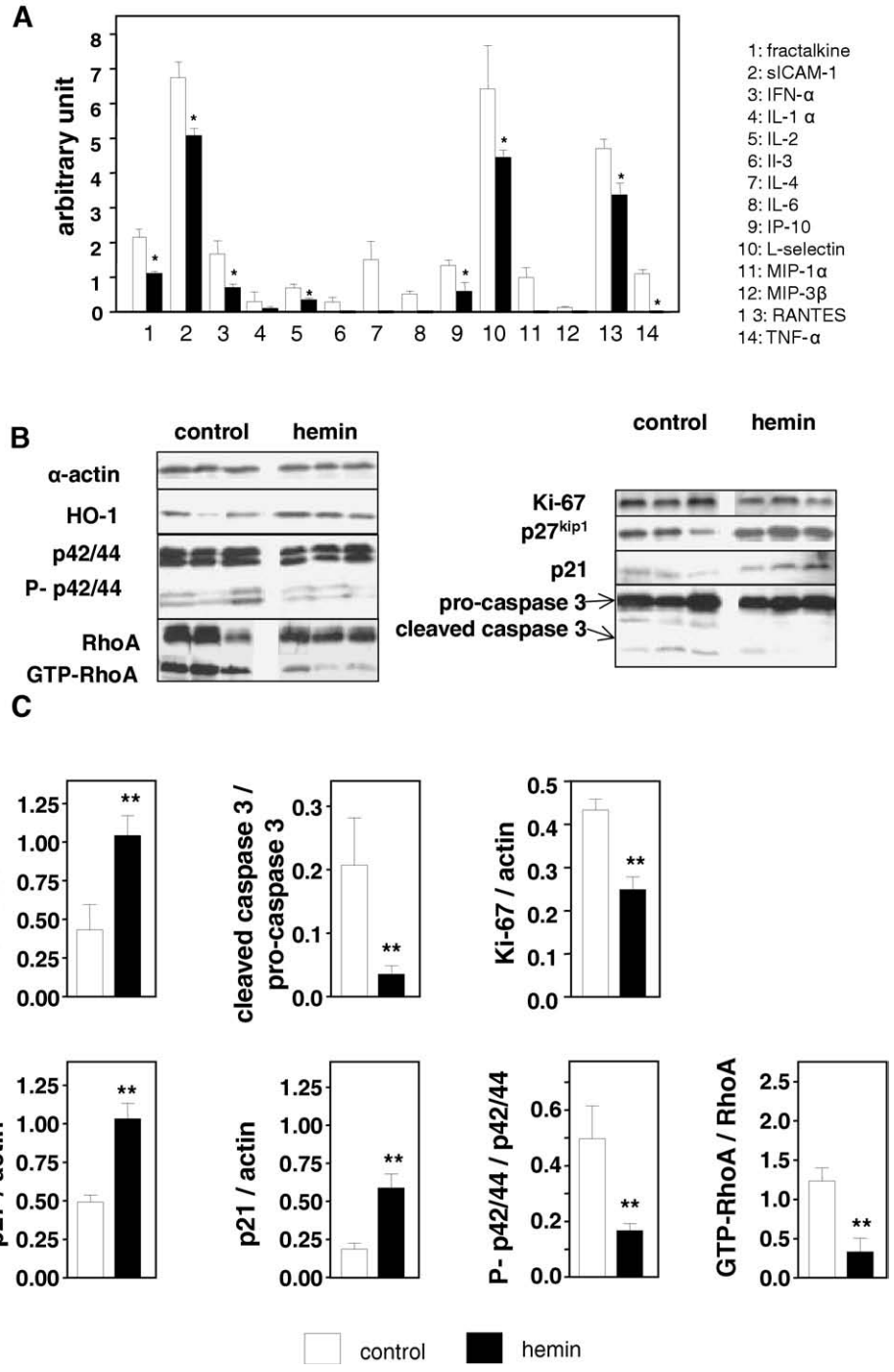

Fig 4. Effect of hemin treatment on protein expression in rat aortas 7 days after stenting. **A,** Protein array of inflammatory cytokines in control (n = 4) and hemin-treated (n = 4) rats. The results are the mean ± standard error of the mean. **B,** Typical Western blot of proteins from stented aortas of control and hemin-treated rats. Each lane represents one rat. **C,** Protein levels were quantified by densitometry and normalized with respect to α-actin. Activities of p42/44 and RhoA were expressed as the ratio of phosphorylated p42/44 to total p42/44 and the ratio of guanosine triphosphate *(GTP)*-bound RhoA to total RhoA, respectively. The results are the mean ± standard error of the mean (n = 5 each group). Significant difference from control: $*P < .05$, $**P < .005$. *HO-1,* heme oxygenase-1; *IL,* interleukin; *INF*α*,* interferon-α; *IP-10,* interferon-inducible protein 10; *MIP,* macrophage inflammatory protein; *RANTES,* regulated upon activation normal T-cell expressed and secreted; *sICAM,* soluble intercellular adhesion molecule 1; *TNF,* tumor necrosis factor.

We next examined the expression of key regulators of cell proliferation. Hemin treatment resulted in significantly increased expression of cyclin-dependent kinase inhibitors p21 and $p27^{kip1}$, supporting reduced proliferation of vascular cells (Fig 4, *C*). Finally, we examined the expression of RhoA and p42/44 MAPK, two proteins that are known to participate in cell proliferation, migration, and differentiation. The RhoA-activated form, guanosine triphosphate (GTP)-bound RhoA, and the expression level of phosphorylated p42/44 were significantly reduced in hemin-treated rats.

Interestingly, we further assessed the effect of hemin on these molecules at day 28 (Fig 5). As expected, HO-1 expression remained higher in hemin-treated rats compared with the controls (n = 5 each; Fig 5, *B*). As previously observed, the protein Ki-67 remained higher ($P < .05$) in the hemin-treated group, although the difference was less than that at day 7 (Fig 5, *C*). Regarding the regulators of cell cycle, p21 ($P < .05$) and $p27^{kip1}$ ($P > .05$) remained up-regulated and GTP-RhoA and phosphorylated p42/44 both remained down-regulated ($P < .05$) in the hemin-treated rats (Fig 5, *C*).

Effect of SnPPIX in a rat model of in-stent stenosis. To ensure that the protective effects of hemin relied specifically on HO-1, we performed additional experiments to test the effect of SnPPIX, a potent competitive inhibitor of HO. In rats treated (28 days) with IP SnPPIX (50 mg/kg) plus hemin (50 mg/kg) for 48 hours, histologic analysis showed that the n/m ratio was increased by 20% (n = 5, $P < .01$) compared with the control groups (Fig 6, *A*). Similarly, long-term treatment with SnPPIX alone increased the n/m ratio by 19% compared with the controls (n = 5 each, Fig 6, *A*). To confirm the inhibitory effect of SnPPIX on HO-1 activity, we assessed plasma bilirubin level. In both the SnPPIX and SnPPIX plus hemin groups, plasma bilirubin levels were similar to those observed in control rats and were significantly lowered compared with the hemin group (Fig 6, *A*).

Effect of CORM-3 in rat model of in-stent stenosis. Because heme catabolism leads to CO production, we tested the effect of CORM-3, a specific CO donor in ISS. In rats treated with IP CORM-3 (30 mg/kg), the n/m ratio measured at 28 days was significantly less than that in the control rats (0.58 ± 0.01 vs 0.70 ± 0.02, n = 5 each, $P < .001$; Fig 6, *A*). This effect of CORM-3 was independent of HO-1 activity, as indicated by the plasma bilirubin level (Fig 6, *A*).

DISCUSSION

Despite the beneficial effects of DESs in reducing the incidence of ISS, concerns have been raised about the associated risk of late stent thrombosis due to the cytostatic and cytotoxic effects on endothelialization of the coated drugs.[5] The results of the present study show that hemin, a potent HO-1 inducer, limited ISS without compromising the endothelialization of the stented arteries in both rat and hypercholesterolemic rabbit models of ISS.

Long-term hemin treatment reduced ISS in the stented rat aortas by 31% and by 48% in the rabbit iliac arteries, a classically used model to test antistenosis therapies.[18] Interestingly, in both hemin-treated rats and rabbits, the BMS struts remained covered. This is the most powerful surrogate indicator of endothelialization and is in contrast to aorta and iliac arteries stented with sirolimus DESs. It is noteworthy that proper healing of stented vessels requires re-endothelialization to limit smooth muscle proliferation and also to ensure proper hemostatic properties. Re-endothelialization of hemin-treated rat aorta and rabbit iliac artery was further supported by electron microscopy analysis and was similar in the control and hemin-treated groups. This effect is in agreement with the multifaceted effects of hemin, which are cell type-specific. Hemin has been reported to limit the proliferation of vascular smooth muscle cells and to promote the proliferation of endothelial cells in vitro.[10,11,19]

Using the rat aorta stenting model, we further investigated the cellular mechanisms underlying the protective effect of hemin. Inflammatory responses and apoptosis have been recognized to be involved in the early events of stenosis.[20-22] We observed that hemin prevented early inflammatory response, in agreement with its well known anti-inflammatory effect.[23] In addition, apoptosis response, assessed by the level of cleaved caspase 3, the end effector of cellular apoptosis, was decreased by 80% after hemin treatment. The reduced apoptosis suggests that the reduced thickness of the neointima was due to reduced proliferation.

In this regard we obtained several lines of evidence of the early antiproliferative effect of hemin. First, the expression of Ki-67, a protein strictly associated with cell proliferation, was significantly lowered in hemin-treated rats. Furthermore the expression of p21 and $p27^{kip1}$ proteins, two cyclin-dependent kinase inhibitors,[24] was higher. Second, the level of activated p42/44 and activated RhoA, two regulators of proliferation, migration, and differentiation of the smooth muscle cells,[25,26] were decreased in hemin-treated rats. The antiproliferative effects were maintained for a longer period (up to 28 days). A similar antiproliferative effect of hemin was reported in animal model of angioplasty.[13,14]

Our observations, however, extend the previous findings in two important ways. First, long-term treatment with hemin limits inflammatory response and apoptosis in an in vivo model of stent-induced vascular injury and limits vascular smooth muscle cell proliferation. Second, to the best of our knowledge, this is the first time that the antiproliferative effects of hemin have been linked to decreased activity of RhoA and increased expression of both p21 and $p27^{kip1}$. Further studies of messenger RNA (mRNA) expression could provide greater insight in the time-course of gene expression during restenosis and in the cellular mechanisms involved in the protective effect of hemin.

In the present study we assessed the expression of proteins at day 7 and day 28, which is within a time frame that allows protein up-regulation or down-regulation. Fur-

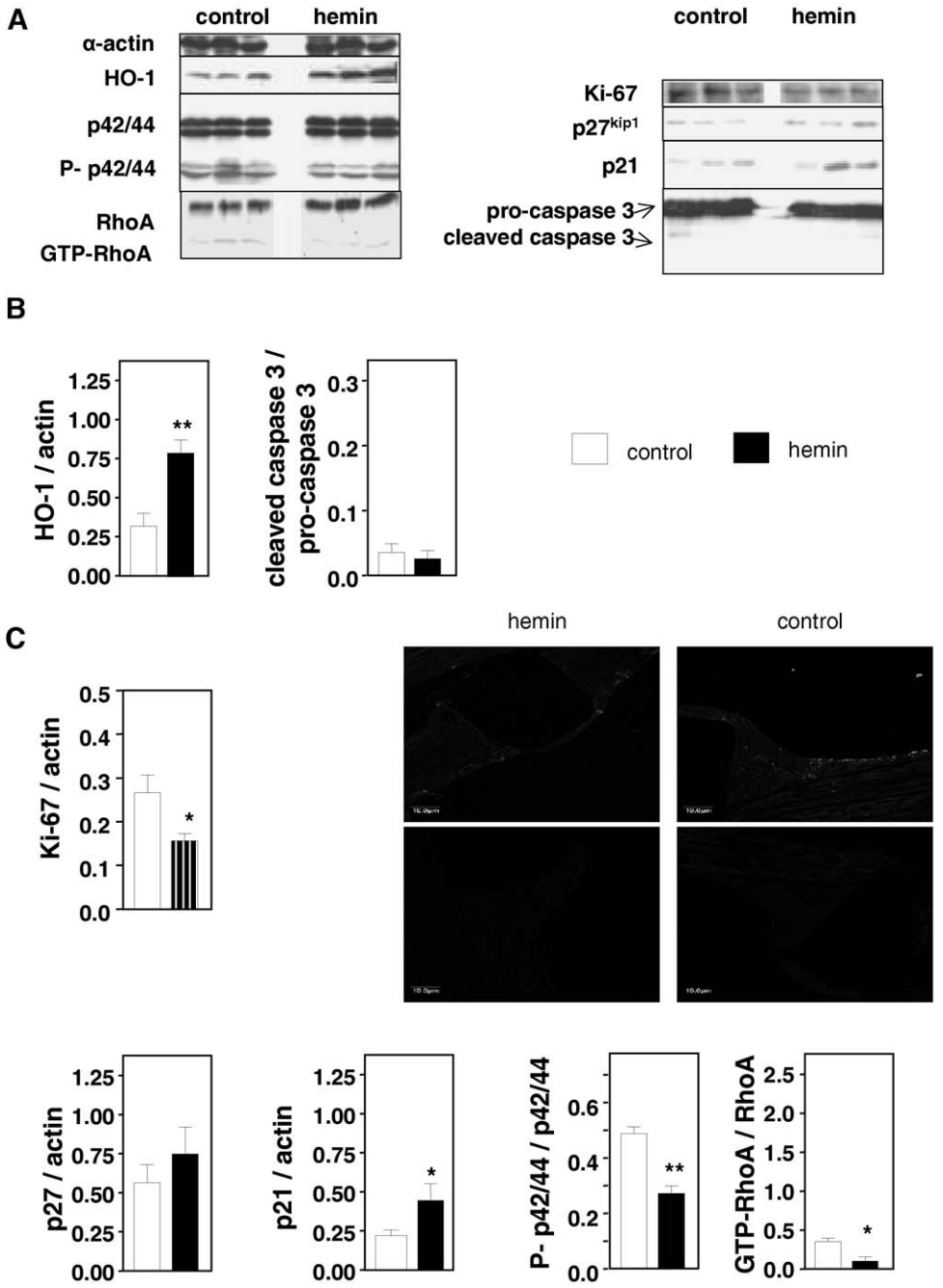

Fig 5. Effect of hemin treatment on protein expression is shown in rat aortas 28 days after stenting. **A,** In this typical Western blot of proteins from stented aortas of control and hemin-treated rats, each lane represents one rat. **B,** Densitometry analysis shows heme oxygenase-1 (HO-1) expression and cleaved caspase 3. **C,** Protein levels are shown for the regulators of cell proliferation. The **upper panel** shows the expression of the Ki-67 protein, assessed by Western blot analysis *(left)* and immunofluorescence *(right)*. Immunofluorescence shows better expression of the Ki-67 protein in the control group. Negative controls are presented below. The **lower panel** shows the expression of the p21, $p27^{kip1}$, p42/44, and RhoA. The results are the mean ± standard error of the mean of five rats in each group. Significant difference from control: $*P < .05$, $**P < .005$. *GTP,* Guanosine triphosphate.

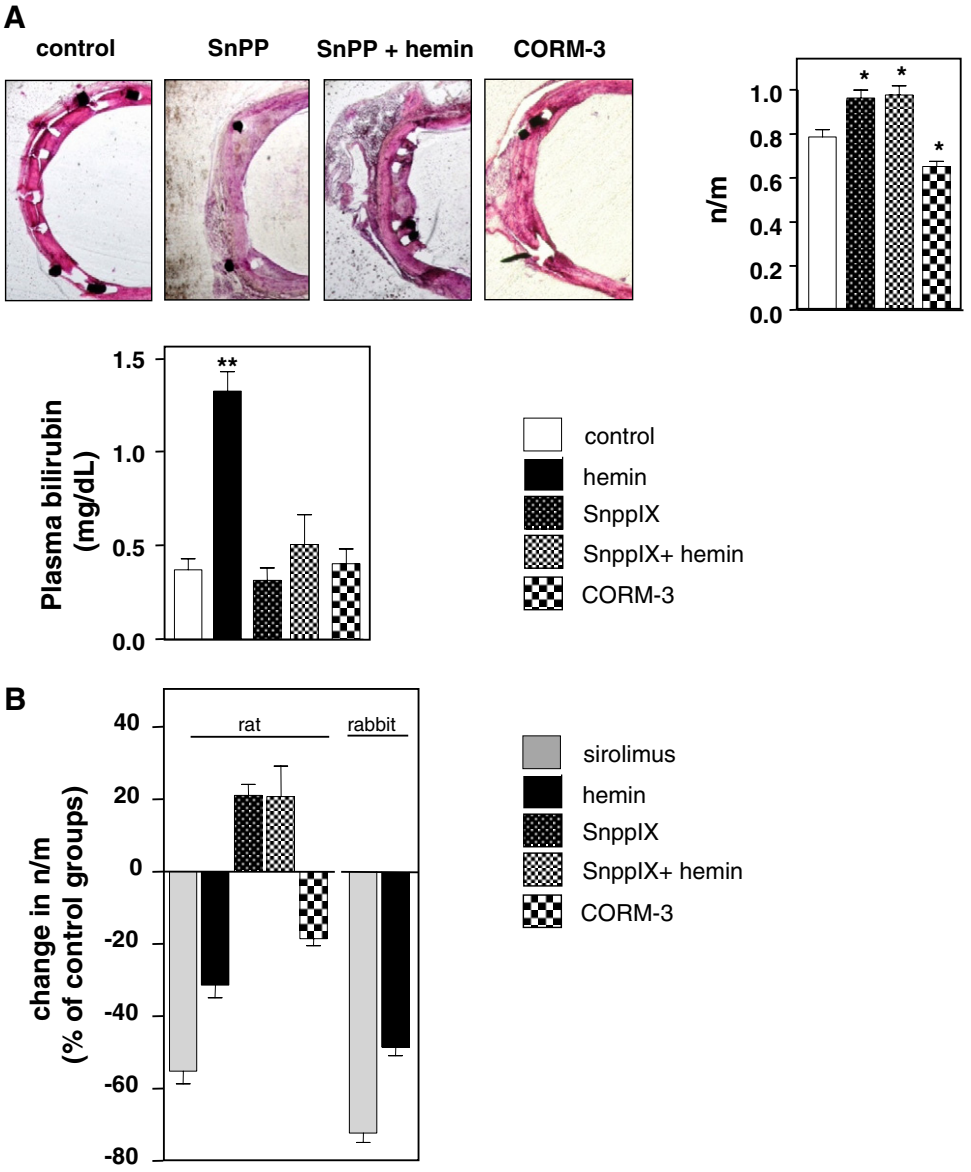

Fig 6. Effect of chronic treatment with tin-protoporphyrin IX *(SnPPIX)* and tricarbonylchloro(glycinato)-ruthenium(II) *(CORM-3)* on in-stent stenosis in rat aorta. **A,** Aorta morphology 28 days after stenting in the control, SnPPIX (15 mg/kg), SnPPIX plus hemin (50 mg/kg, each), and CORM-3 rats (30 mg/kg; n = 5 all groups). The bar graph represents mean n/m ± standard error of the mean. The plasma bilirubin levels in the different groups are presented below. Significant difference from control: $*P < .05$, $**P < .005$). **B,** Effect of pharmacologic treatments on in-stent stenosis in rat aortas and rabbit iliac arteries. Results are expressed as percentage change of n/m when compared with control groups. The results are the mean ± standard error of the mean.

thermore, analysis of mRNA expression would have been useless regarding the effect of hemin treatment on the activation of the proteins such as p42/44, RhoA, and caspase 3, which relies on phosphorylation, GTP binding, or cleavage.

Having established that hemin induced HO-1 expression and limited ISS, we wanted to verify that this effect relied on the HO-1 activity. In presence of SnPPIX, a potent HO-1 inhibitor, hemin no longer had an effect, as indicated by the increased stenosis (+20%). SnPPIX alone worsened stenosis, suggesting that endogenous HO might also limit ISS. In this regard, recent studies demonstrated a greater occurrence of in-stent coronary stenosis associated with a *ho-1* gene promoter polymorphism,[27,28] although another report cast doubt on this association.[29]

We further assessed the activity of HO-1 by measuring the byproducts of heme degradation, such as CO and bilirubin. With blood gas analysis we failed to detect any

difference in CO production. However, we observed that plasma bilirubin production was increased in hemin-treated rats and lowered in the presence of SnPPIX. These data further supported the conclusion that increased HO-1 activity limited ISS.

CO and bilirubin have both been proposed to exert protective actions on the cardiovascular system.[30] Interestingly, the levels of bilirubin in the groups of rats treated with SnPPIX were similar to those in control rats, despite higher stenosis. Therefore, a byproduct of heme catabolism, possibly CO, might afford protection against ISS. This conclusion was reinforced by the decreased n/m ratio in CORM-3-treated rats, in agreement with the anti-inflammatory, antiproliferative, and antiapoptotic effects of both CO gas and CORMs.[17,31-34] Thus, in our model, it is conceivable that the hemin-afforded protection relies on both CO and bilirubin.

This study has some limitations. First, in the rat model of in-stent stenosis, the neointima hyperplasia is very low compared with the well-established porcine coronary artery and rabbit iliac artery models. Although the low n/m ratio weakens this animal model, we observed a reliable and reproducible n/m ratio among the different control rats. Most importantly, after sirolimus-DES deployment, the n/m ratio was significantly decreased by 55%. Thus in the rat abdominal stenting model, the n/m ratio is robust and significant enough to assess the effect of drug therapy that can reduce in-stent stenosis.

Second, hemin treatment was initiated 48 hours before stenting. This delay was necessary to increase HO-1 after the IP injection of hemin. However, such approach would not be compatible with an emergency clinical situation such as acute ST elongation myocardial infarction, It remains to be determined if local delivery of hemin or CO, or both, through the means of release platforms (such metallic or bioresorbable DESs) would allow faster up-regulation of HO-1. In this regard it is noteworthy that in cultured smooth muscle cells, hemin induced HO-1 up-regulation ≤4 hours.[10,35] Furthermore, local delivery should be likely important to avoid any potential systemic side effect.

Third, the protective effect of hemin against ISS will require long-term follow-up to determine (1) if the protection is maintained at later time points (3 to 6 months) and (2) if there is any rebound in inflammatory and proliferative response when hemin treatment is stopped.

Finally, we observed that the decreased n/m was less in hemin-treated rats than that in aortas stented with sirolimus DESs. However, sirolimus inhibits mammalian target of rapamycin C1 and C2 and ultimately increases the cyclin-dependent kinases (CKDs) inhibitors in endothelial cells and impairs their viability and function,[36] whereas HO-1 exerts cytoprotective effects on endothelial cells and increases their proliferation through the down-regulation of cyclin-dependent kinases.[37]

CONCLUSIONS

Despite these limitations, the present study shows that hemin induced HO-1 expression and reduced in-stent stenosis in rat and hypercholesterolemic rabbit models. Cellular analysis revealed that hemin limited the early inflammatory response, apoptosis, and the proliferation of vascular cells. Most importantly, hemin did not alter the healing and re-endothelialization of the stented arteries, which contrasts with the findings obtained with the classic sirolimus DES. Therefore, HO-1 activation appears to be important in limiting ISS and can be regarded as new therapeutic target.

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AUTHOR CONTRIBUTIONS

Conception and design: JMH, PL
Analysis and interpretation: JMH, BM
Data collection: JMH, BM, RU
Writing the article: JMH
Critical revision of the article: JMH, RU, RM, PL
Final approval of the article: JMH, BM, RU, RM, PL
Statistical analysis: JMH, BM
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Overall responsibility: JMH
JMH and BM contributed equally to this work.

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